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Creation of Transgenic Sugar Beet Lines Expressing Insect Pest Resistance Genes *cry1C* and *cry2A*

D. I. Lytvyn[a], V. V. Syvura[a], V. V. Kurylo[a], V. D. Olenieva[b], A. I. Yemets[a], and Ya. B. Blume[a]

[a] *Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, ul. Osipovs'kogo 2A, Kyiv, 04123 Ukraine*

[b] *Taras Shevchenko National University of Kyiv, ul. Volodymyrska 64/13, Kyiv, 01601 Ukraine*

e-mail: ldmi@ukr.net

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Abtract—The impact of insect pests significantly limits sugar beet crop yields. The integration of *cry*-genes of *Bacillus thuringiensis* into the plant genome is a promising strategy to ensure plant resistance. The aim of this work was to obtain sugar beet lines (based on the MM1/2 line) transformed with *cry2A* and *cry1C* genes. We have optimized the transformation protocol and direct plantlet regeneration protocol from leaf explants using 1 mg/L benzylaminopurine as well as 0.25 mg/L benzylaminopurine and 0.1 mg/L indole-butyric acid. Consequently, transgenic sugar beet lines transformed with vector constructs pRD400-*cry1C* and pRD400-*cry2A* have been obtained. PCR analysis revealed integration of *cry2A* and *cry1C* into the genome of transgenic lines and expression of these genes in leaf tissues was shown by reverse transcription PCR.

Keywords: *Beta vulgaris*, *cry*-genes, genetic transformation

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INTRODUCTION

Besides sugar cane, cultivated crop sugar beet (*Beta vulgaris* L.) has a key role in the production of sugar and provides raw material for 70% of its global production. This biannual crop is suitable for cultivation in temperate climates, and today approximately 40 countries are involved in its commercial cultivation [1]. In addition, due to its high biomass growth, sugar beet is viewed as a promising source of synthesis and accumulation of new metabolites in roots [2]. Moreover, due to the high energy value of intermediates of sugar beet processing, it can be used as an effective raw material for bioethanol production [3–5]. However, the biological characteristics of sugar beet, namely cross-pollination and a biannual development cycle, combined with a high level of heterozygosity, makes the process of production of new varieties via classical breeding techniques long. In addition, a significant loss of culture is due to adverse environmental effects, various diseases, and pests. To date, more than 250 pests of sugar beet are known. The most common and harmful are: miner flies, sod webworms, sugar beet weevils, caterpillars of owl-moth, miner moths, and a number of mites and nematodes. Globally, the potential damage caused by pests, can reach up to 80% of the yield [6]. In this regard, and also due to the significant financial costs associated with the use of insecticides, it is advisable to create varieties and lines of sugar beet resistant to parasites. One of the most effective strategies to combat pests is the use of the toxic properties of cru-proteins of gram-positive pathogenic bacterium *Bacillus thuringiensis*. Cry-proteins (or Bt-proteins) of different families exhibit highly specific toxicity against representatives of specific insect orders. For example, *cry1* and *cry9* genes encode proteins toxic for *Lepidoptera*, *cry2* encode proteins toxic for *Diptera* and *Lepidoptera*, *cry3* encode proteins toxic for *Coleoptera*, and *cry4* and *cry11* encode proteins toxic for *Diptera* [7, 8]. Thus, the production and use of genetically modified plant varieties expressing certain toxic proteins of this group are designed to increase crop yields and reduce the negative environmental impacts associated with the use of insecticides [9–12]. The mechanism of the cytotoxic action of cry-proteins occurs in the intestine of insects after proteolysis of prototoxins and formation of active 5-endotoxins. The effect of 5-endotoxin, after its binding to a specific membrane receptor, leads to perforation of intestinal epithelial cells and the death of the insect. The nontoxicity of cry-proteins on human and other vertebrate cells, as well as plant cells, also should be taken into consideration [13].

Large-scale implementation of genetic engineering approaches for the production of sugar beet varieties and lines resistant to pests is prevented by the complexity of this culture under in vitro conditions. Despite extensive data demonstrating the morphogenetic activity of isolated tissues and organs of sugar beet, its regenerative potential largely depends on the genotype and variety of a parent plant. For example, the highest results of regeneration were demonstrated

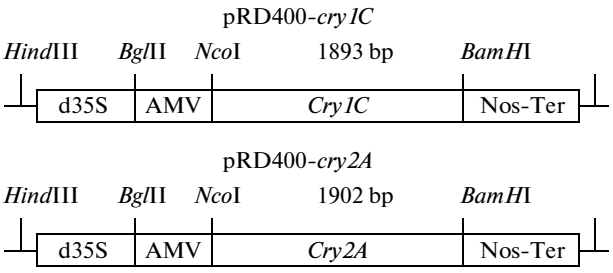

Fig. 1. Schemes of genetic constructs based on the binary vector pRD-400: d35S—twice amplified 35S promoter of cauliflower mosaic virus; AMV—amplifier of translation of alfalfa mosaic virus; Nos-Ter—NOS terminator.

for tetraploid forms of sugar beet, while triploid and diploid forms have lower and lowest results, respectively. The regenerative capacity of the culture is affected by both the type and age of the original tissue and culture conditions in vitro, including the availability and the ratio of certain growth regulators [14–18]. Thus, biotechnological improvement of *B. vulgaris* requires further improvement of optimization methods for its microclonal breeding and efficient plant regeneration from in vitro culture.

Development of methodological approaches for genetic transformation of sugar beet is being carried out in several directions. The most traditional and common method is based on Agrobacterium-mediated transformation using *Agrobacterium tumefaciens* and *A. rhizogenes* [19–21]. However, despite the very high sensitivity to infection by *Agrobacterium*, the production of transgenic regenerates of sugar beet are largely dependent on plant genotype and bacteria strain [19]. To date, optimal protocols for the Agrobacterium-mediated transformation of *B. vulgaris* have been developed that are based on the varietal characteristics of this culture [22–26]. Another modern approach for the production of transgenic sugar beet lines is the use of biolistic bombardment of cells or tissue using microparticles. Positive results were achieved by this approach for the transformation of suspension culture, embryogenic callus derived from hypocotyl, and chloroplast transformation [21, 27, 28]. At the same time, despite the considerable relevance of obtained sugar beet lines with resistance to pests, which was determined by products of *cry*-genes, global experience in this area is limited. Among the few works, the production of sugar beet lines transformed with *cry1Ab* gene [29] and lines expressing *cry-1Ab* and *cry1C* that exhibit resistance to lepidopterans [30] was described. Therefore, the goal of this study was to optimize the protocols of regeneration of sugar beet and production of transgenic lines of this culture expressing *cry2A* and *cry1C* resistance genes.

MATERIALS AND METHODS

Plant material. The starting material in the experiments for optimization of regeneration protocols and Agrobacterium-mediated transformation was the parent breeding line MM1/2 (breeding pollinator during heterosis breeding) of sugar beet (*Beta vulgaris* L.), kindly provided by the Institute of Bioenergy Crops and Sugar Beet of the National Academy of Sciences of Ukraine.

In vitro transformation and regeneration. MS agar medium was used as the basic medium [2] for plant cultivation and regeneration, and a modified MS medium containing 1 mg/L benzylaminopurine (BAP) was used for shoot regeneration. The regeneration was induced by placing explants (leaf disks with diameter of 10–15 mm) on the regeneration medium, and culturing was performed under diffuse illumination with a 16-hour light period. Rooting of the regenerated shoots was induced by transferring to MS medium containing 0.5 mg/L α-naphthylacetic acid (NAA).

For Agrobacterium transformation, the *Agrobacterium tumefaciens* LB 4440 strain and vector constructs pRD400-*cry1C* and pRD400-*cry2A*, containing *cry1C* and *cry2A* respectively, kindly provided by prof. I. Altosaar (University of Ottawa, Canada), were used (Fig. 1).

Agrobacterium-mediated transformation was carried out according to Norouzi et al. [24] with some modifications. Leaf disks with a diameter of 10 mm were used as explants. *A. tumefaciens* LB 4404 strains containing binary constructs were grown in liquid LB medium containing 50 mg/L kanamycin. Agrobacterium culture, which reached an optical density (OD_{600}) of 0.5 was pelleted and resuspended in MS containing 50 mM acetocyringone (Sigma-Aldrich, United States), followed by incubation for 5 hours. Cuts were made of the explants before transformation for successful infection and explants were incubated with Agrobacterium suspension for 5 minutes with stirring. Subsequently, remnants of bacterial suspension were removed from explants and they were cultured on MS agar medium for 3 days. Then samples were washed twice for 30 min in sterile water containing 500 mg/L cefotaxime, and plated onto a regeneration medium containing selective agent kanamycin (200 mg/L) and cefotaxime (300 mg/L) for the elimination of excess of Agrobacterium. After 2 weeks, the explants were transferred onto a fresh medium. Subsequent passages with two-week intervals were performed on the medium with decreased concentration of antibiotics (100 and 250 mg/L for cefotaxime and kanamycin, respectively).

Molecular biological analysis. DNA extraction and PCR analysis of transformants was performed using Thermo Scientific Phire Plant Direct PCR Kit no. F-130 (Thermo Fisher Scientific Inc., United States) according to the manufacturer's recommendations. The following pairs of primers were used: 5'-GGTTG-

TAGGAGTTGCCGTTGC-3' (R) and 3'-CGCA-GTTCCAGATCCAGGGCTA-3' (F) for the *cry2A* gene and 5'-AACCTGTGGGAGAATCCTTGCCTG-3' (R) and 3'-TCTATGGAACTATGGGAAACGCCG-CTC-3 (F) for the *crylC* gene with amplification of PCR product with sizes of 280 bp and 1149 bp, respectively. The reaction was carried out in thermocycler PCR Applyed Biosystem 2720 (United States) for 40 cycles under the following conditions: denaturation at 98°C (5 s), annealing at 66°C for *crylC* and 62°C for *cry2A* (5 s), synthesis at 72°C (20 s), and final elongation at 72°C (5 min).

RNA for RT-PCR was isolated using reagent TRIzol Reagent (Life Technologies Co., United States). Reverse transcription was performed using Thermo Scientific RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc., United States) according to the manufacturer's protocol and recommendations. cDNA synthesis was performed on a matrix of total RNA (0.5 mg) for 1 h at 42°C in a 20 μL reaction mixture containing 20 pmol of gene-specific primers (5'-GGTTGTAGGAGTTGCCGTTGC-3' and 5'-AAC-CTGTGGGAGAATCCTTGCCTG-3' for *cru2A* and *cru1C*, respectively) and 200 U of reverse transcriptase. After the reaction, the samples were incubated for 10 min at 70°C for inactivation of reverse transcriptase. For the electrophoretic separation of PCR products, 2% agarose gel based on a 1XTris-borate buffer was used.

RESULTS AND DISCUSSION

Selection of conditions for regeneration and genetic transformation. One of the main prerequisites for the production of transgenic crops in vitro is the efficient and reproducible procedure for the regeneration of transformants from original tissue explants. Due to the complexities associated with optimizing the regeneration of sugar beet, which may be due to the varietal and genotypic features, work for the selection of regeneration conditions of plants from leaf discs of MM1/2 line of *B. vulgaris* was performed earlier. The use of a culture medium containing 1 mg/L BAP provided the optimal performance of plant regeneration in the selection line. Plant regeneration (percentage of obtained regenerants from the total number of planted explants 10–14 days after planting) on this medium was 60%. Comparative studies demonstrated that the most suitable explants for induction of direct regeneration of plants are in the leaf discs of sugar beet. The experience of other laboratories demonstrates the effective application of cytokinins, in particular BAP, as inducers of sugar beet regeneration in vitro [14, 32–34]. Application of phytohormone concentrations not lower than 1 mg/L are usually effective for the direct regeneration from explants of leaves, petioles, stems and callus. Similar results were obtained in our experiments with the MM1/2 line, where 1 mg/L BAP was added in culture medium for the induction of direct

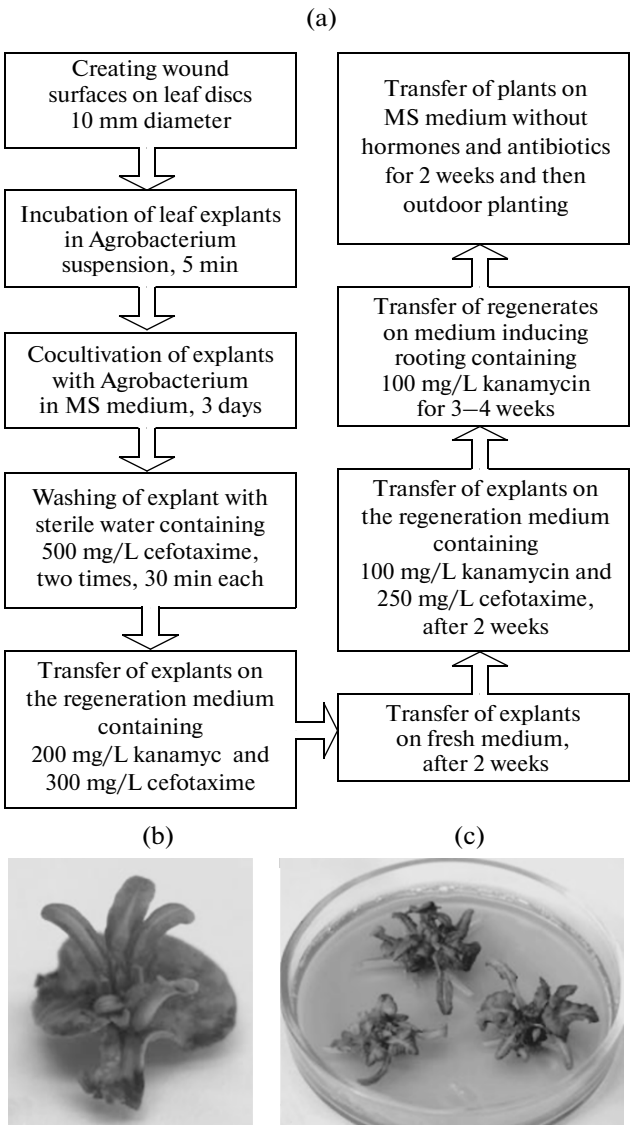

Fig. 2. Stages of transformation and regeneration of transgenic shoots of sugar beet: (a) general scheme of production of transgenic sugar beet lines transformed with *cry2A* and *cry1C* genes, (b) shoot regeneration in the area of central vein of leaf explants, (c) 4-week-old transformed shoots before transfer onto the medium for rooting.

shoot regeneration from leaf explants. It should be noted that in many cases the combined use of cytokines and auxins was also effective and that effective working concentrations of used phytohormones were significantly reduced during combined use. In previously published works quite effective application of combination of BAP and NAA [35], as well as BAP, IAA and 2,3,5-triiodobenzoic acid [28] was demonstrated.

Agrobacterium-mediated transformation of sugar beet was performed according to the method [23] with some modifications related to the size and type of explants, concentrations of selective agents and the

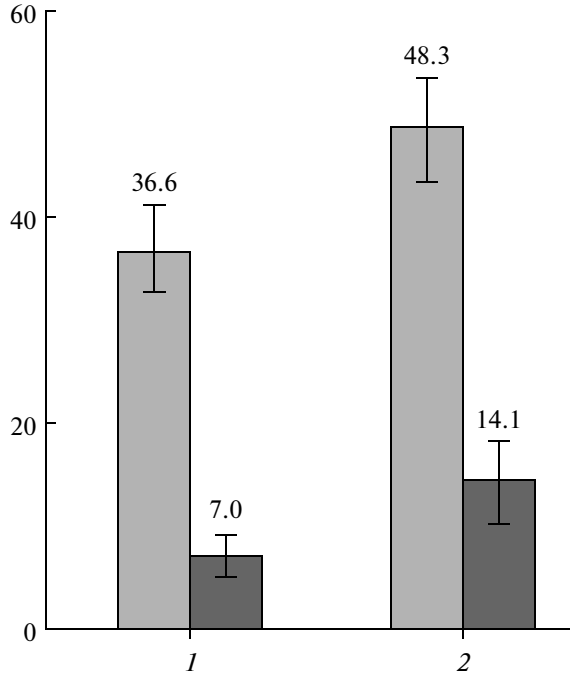

Fig. 3. Indicators of genetic transformation efficiency of sugar beet by (*1*) pRD400-*cry2A* and (*2*) pRD400-*cry1C* constructs: ▢ percentage of explants with signs of shoot regeneration after 4 weeks of cultivation on selective media; ■ percentage of explants on which plants were formed 6 weeks after transformation and selection. The average result of three experiments.

duration of cultivation of the material on selective media (Fig. 2a). Regenerating shoots were observed on the surface 2 weeks after explants were transplanted on regeneration medium containing 200 mg/L kanamycin and 300 mg/L cefotaxime.

In most cases, the formation of regenerated occurred in the area of wound surfaces of the central vessels (Fig. 2b), and normally formed shoots ready for further rooting were obtained 4–6 weeks after transformation (Fig. 2c). Efficiency of genetic transformation by *cry1C* and *cry2A* genes was different. In order to establish this value, the percentage of regenerates selected after transformation on selective medium to the total number of explants planted was determined. The following values were examined and compared: the total number of explants, the number of explants with signs of recovery when grown for 4 weeks on selective medium with 200 mg/L kanamycin, and the number of explants with plant regeneration on medium with 100 mg/L kanamycin 6 weeks after transformation. Sufficiently high yield of transgenic plants with slight superiority of transformation efficiency in case of *cry1C* gene was demonstrated as a result of the transformation with both genetic constructs (Fig. 3).

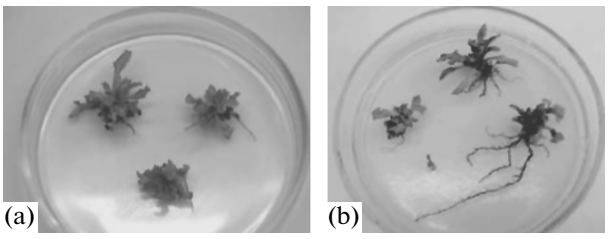

Fig. 4. Root formation in transformed sugar beet plants: (a) formation of roots on the medium containing 0.5 mg/L NAA, (b) subsequent morphogenesis of roots on MS medium without hormones.

Selection of conditions for rooting transformants. The final step is the selection of optimal conditions for rooting sugar beet plants. As in Norouzi [24], optimization of the medium for rooting was performed by selection of the effective concentration of NAA. The concentration of NAA 0.5 mg/L was most suitable in the case of the MM1/2 line. The effectiveness of rooting was 80% 3–4 weeks after planting shoots on the above-mentioned medium (Fig. 4a).

The remarkable fact is that, according to Bekheet et al. [35], the use of NAA led to mediocre results, while the medium effective for rooting contained indolebutyric acid. Such disparities of effectiveness of different phytohormones for the induction of regeneration and rooting in vitro can be logically explained by previously mentioned genetic variability of sugar beet. Due to this, we can assume the need to adapt protocols for regeneration of sugar beet in vitro during the transformation of different lines and varieties. In addition, rooting of plants also occurred spontaneously on the MS medium without hormones, but the percentage of rooted plants was low. With root lengths of approximately 20 mm, plants were trans-

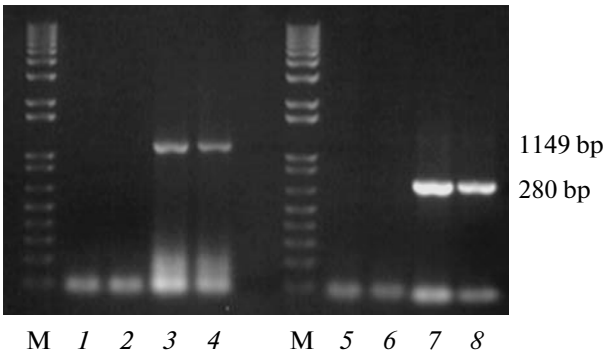

Fig. 5. Results of PCR analysis of transformed sugar beet plants (amplification of *cry2A* and *cry1C* gene fragments): (*1*) control (amplification without DNA template with primers for *cry2A* gene), (*2*) amplification of *cry2A* gene in control untransformed plant of MM1/2 line, (*3*) amplification of plasmid *cry2A* gene in pRD400-*cry2A*, (*4*) amplification of *cry2A* gene in plant transformed with pRD400-*cry2A* plasmid, (*5*) control (amplification without DNA template with primers for *cry1C* gene), (*6*) amplification of *cry1C* gene in control untransformed plant of MM1/2 line, (*7*) amplification of plasmid *cry1C* gene in pRD400-*cry1C*, (*8*) amplification of *cry1C* gene in plant transformed with pRD400-*cry1C* plasmid.

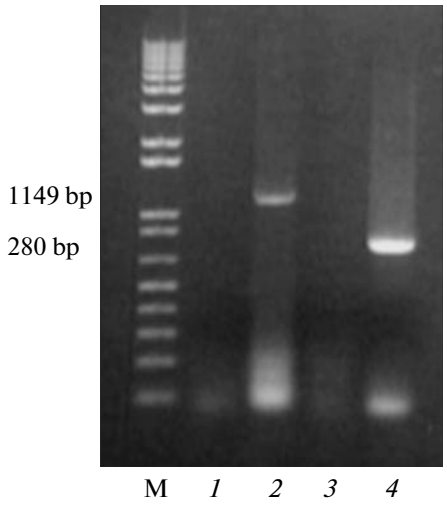

Fig. 6. Results of electrophoretic analysis of cDNA amplification products of transformed sugar beet plants: (*1*) expression of *cry2A* gene in control plant, (*2*) expression of *cry2A* gene in the transformed line, (*3*) expression of *cry1C* gene in control plant, (*4*) expression of *cry1C* gene in the transformed line.

ferred for 2 weeks to an MS medium without hormones for formation and adaptation of the root system to outdoor growing (Fig. 4b).

Molecular genetic analysis. The ability of transformants to develop over a long time in the presence of selective agents is not conclusive proof of the transgenic nature of the obtained plants. In order to verify the integration of genes of interest into the plant genome, we performed PCR analysis of transformed sugar beet lines regenerated on the selective medium using primers specific to the *cry1C* and *cry2A* genes. As a result, the presence of the studied genes in the transformed plants was established (Fig. 5, data presented for some lines).

Isolation of RNA from transformants for production of cDNA via reverse transcription reaction and amplification of *cry1C* and *cry2A* genes was performed in order to confirm not only insertion of genes into the plant genome but also their efficient expression. The result confirmed the expression of these genes. It should also be noted that the level of *cry1C* gene expression was much higher than that of *cry2A* gene (Fig. 6).

Thus, we obtained transgenic sugar beet lines expressing *cry2A* and *cry1C* genes, with potential resistance to insect pests of *Lepidoptera* and *Diptera*. The success in the use of the properties of cry-proteins for the production of pest-resistant plants so far is obvious. Among the cultures whose transformation by *cry*-genes was successful are potato [36, 37], tomato [38–40], rice [41, 42], maize [43, 44], and sweet potato [45]. The amount of research in this area devoted to sugar beet is not enough for its effective cultivation corresponding to modern requirements of growing. The lines obtained in this research, can be used as a basis for the production of new sugar beet varieties resistant to pests.

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